Developing and analysing novel tools to study endogenous WNT signalling in mice

van de Moosdijk, A.A.A.

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Chapter 6

Generation of an *mTurquoise2-Ctnnb1* knock-in mouse via CRISPR/Cas9 genome editing

Anoeska van de Moosdijk¹, Saskia de Man¹, Lona Kroese², Lotte Hofstee¹, Yorick van de Grift¹, Ivo Huijbers² and Renée van Amerongen¹

¹ Swammerdam Institute for Life Sciences, Section of Molecular Cytology, University of Amsterdam, The Netherlands
² Mouse Clinic for Cancer and Aging (MCCA) Transgenic Facility, The Netherlands Cancer Institute, Amsterdam, The Netherlands
Abstract

The use of genetically engineered mouse models has long been the gold standard for studying gene and protein function in mammals. Usually, transgenic or knockout mice are generated to overexpress or remove a protein of interest. However, knock-in mice can also be designed to leave endogenous gene expression intact. For instance, to monitor activity of a specific signal transduction pathway, a fluorescent reporter gene can be inserted into the locus of a direct target gene of the pathway under study. If this knock-in is designed to be expressed in frame with the gene of interest, the resulting fusion protein acts as a direct reporter, allowing in vivo imaging of the protein in real-time.

Beta-catenin (CTNNB1) is a key player in the WNT signalling pathway and its main downstream effector. Its precise biological behaviour and signalling dynamics remain unknown. Here we describe the stepwise generation of a CRISPR/Cas9 genome editing toolbox for modifying the endogenous Ctnnb1/CTNNB1 gene in mouse and human cell lines. Moreover, we report the optimization of CRISPR/Cas9-mediated genome editing in zygotes to generate a neutral knock-in of a bright fluorescent reporter into the endogenous Ctnnb1 locus, resulting in the successful generation of an mTq2-Ctnnb1 knock-in mouse. This mouse model serves as a direct in vivo reporter for WNT/CTNNB1 signalling activity. Moreover, mTq2-CTNNB1 also serves as a global marker for epithelial cells.

Introduction

Elucidating the complex and dynamic role of signalling pathways in development and disease requires the use of transgenic model organisms. The use of genetically engineered mouse models has long been the gold standard for studying gene and protein function in mammals. By creating loss-of-function (LOF) or gain-of-function (GOF) mutants, the phenotypic effects of deleting or overexpressing specific factors can be analysed during embryonic development and in postnatal life. Sometimes, however, one wishes to visualize rather than perturb signalling to monitor activity of the pathway under investigation. In that case, a fluorescent reporter gene can be inserted into an endogenous locus. If this knock-in (KI) is designed to be expressed in frame with the gene of interest, the resulting fusion protein acts as a direct reporter, allowing in vivo imaging of the protein in real-time and, if needed, at subcellular resolution. By tagging the Ctnnb1 locus and thereby creating a CTNNB1 fusion with a fluorescent protein, this should allow us to visualize endogenous
WNT/CTNNB1 signalling and to decipher the endogenous molecular behaviour of this important WNT pathway player. One of the advantages of creating an endogenous fluorescent fusion protein, is that it allows the subcellular localization of the endogenous protein to be monitored not only in fixed, but also in living cells and tissues, or the recently developed organoids (Rios and Clevers, 2018).

Traditionally, knock-in mice have been generated via gene targeting in mouse embryonic stem cells (mESCs). This method relies on rare homologous recombination events and requires the screening of hundreds of mESC clones. As a result, this method is time consuming – often taking at least 6 months – and labour-intensive (Capecchi, 2005). Genome editing using clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) shows great promise to overcome this hurdle. Using CRISPR technology, a one-step protocol allows the creation of transgenic mice (Lin et al., 2018; Yang et al., 2013). An injection mix containing all of the required gene-editing components can be injected directly into zygotes, bypassing the need to culture mESCs. The reported efficiencies for creating targeted knock-in mice range from 8.5% to even 100% (Lin et al., 2018; Quadros et al., 2017; Yang et al., 2013), promising a quicker and less laborious method than traditional targeting. Moreover, CRISPR-mediated genome editing also bypasses the need for an antibiotic resistance cassette in the targeting construct, which is typically required for selecting mESC clones. As such, it allows the creation of a completely seamless knock-in allele, eliminating the introduction of unwanted changes that can potentially impact gene expression and thereby affect the phenotypic outcome (as reviewed in (Elison and Acar, 2018)).

Introducing large genomic changes via CRISPR
CRISPR/Cas9 introduces double-stranded breaks (DSBs) in genomic DNA, guided to its destination by a guide RNA (gRNA). In nature, the gRNA is composed of two parts: A small, variable crRNA complementary to the target DNA and a larger, invariant scaffolding RNA molecule, the tracrRNA. For gene-editing purposes, the gRNA is typically supplied as a fusion molecule, known as a single-guide RNA (sgRNA) (Hsu et al., 2014; Jinek et al., 2012).

The Cas protein firstly and mostly used for genome editing is Cas9 from *S. pyogenes*. It requires a 20bp recognition sequence in the DNA (complimentary to the 20bp spacer sequence present in the custom-designed sgRNA) following by a protospacer adjacent motif (PAM sequence), which is any NGG in the genome (Jinek et al., 2012). The DSB is introduced 3bp upstream of the PAM sequence. Repair of this break occurs via non-specific, non-homologous end joining (NHEJ) and results in random insertions and deletions, referred to as indels (Rouet et al., 1994). This faulty repair process is highly suitable for knocking out a protein of interest by disrupting its genetic coding sequence. However, the break can also be repaired via homology-directed repair (HDR), provided that a repair template containing DNA
homology arms—corresponding to the regions flanking the break—is present (Capecchi, 1989). HDR is typically flawless and offers the opportunity to knock-in or modify a DNA sequence of interest at a specific target site with great precision and control, thereby allowing the introduction of point mutations as well as larger genomic insertions.

Different strategies exist to introduce DNA sequences at the desired location. Many of these have been optimized in cell culture and it is unclear if they can be translated directly to in vivo zygote targeting. Despite its first reported use in mammalian cells many years ago (Cong et al., 2013; Mali et al., 2013), application of the technique for the purpose of generating genetically engineered mice is, in many ways, still in its infancy: protocols between labs seem to differ and multiple parameters have been reported to affect targeting efficiency.

First, the gRNA choice plays a large role: not all potential targeting sequences (i.e. any 20 nucleotides followed by an NGG) result in equally efficient, on-target cutting by Cas9. Moreover, gRNAs can also allow cutting at off-target sites, which is not desired because the resulting effects are unknown and difficult to analyse. For any locus, it is therefore key to find a (set of) gRNA(s) with high on-target efficiency, but a low chance for off-target cutting. Prediction algorithms are a great help in ranking the different options. However, these design tools are still actively being developed and improved and ultimately the best way to decide is by empirically testing several gRNAs for each locus of interest. The gRNA should also meet additional requirements. For instance, it should induce cutting close to—preferably within 8bp—the desired genomic integration site, because this greatly enhances HDR efficiency (Liang et al., 2017b).

Second, Cas9 can be introduced into the zygote as either mRNA or protein. The protein leads to higher targeting efficiencies compared to Cas9 mRNA delivery, especially when a Cas9/sgRNA nucleo-protein complex is preassembled before injection (Jung et al., 2017; Ménoret et al., 2015).

Third, the repair construct can be supplied in different ways, depending on the desired modification and targeting efficiency. Repair constructs can be designed to have various homology arm lengths, be single- or double-stranded and be presented as linear or circular DNA, or even as a chromatin template (Cruz-Becerra and Kadonaga, 2020). Many varieties have been suggested with varying efficiency. For example, when introducing small insertions (<200bp), single-stranded donor oligonucleotides (ssODN) are highly efficient repair templates, resulting in up to 80% targeting efficiency for different loci (Yang et al., 2013, 2014). However, creating larger knock-ins remains a challenge with overall lower efficiencies compared to smaller integrations.

Finally, the locus and its genomic context can be crucial for the success of a knock-in attempt. This has long been known from mESC targeting studies, where different loci can have vastly different targeting efficiencies (Hasty et al., 1994;
Müller, 1999). Many initial CRISPR targeting studies were performed using the ROSA26 locus, known for its high targeting efficiency in conventional mESC targeting experiments and typically regarded as a “safe-harbour” locus expressed in nearly all mouse tissues (Friedrich and Soriano, 1991). In this locus, the successful integration of 8kb to 11kb DNA constructs was reported following zygote injection of Cas9/sgRNA components together with circular plasmid DNA repair templates with 1kb 5’ and 0.8-4kb 3’ homology arms, resulting in an HDR rate of 10-20% (Chu et al., 2016). This is higher than reported for most other tested loci using a comparable targeting approach, suggesting that the locus-specific efficiencies might be similar for CRISPR targeting and more conventional HDR-mediated approaches. One reason for the efficient targeting in Rosa26 might be its “open” chromatin structure. Unfortunately, the chromatin structure of a locus of interest is often unknown and, even if known, not easily altered. The GC content of the locus and the absolute number of available gRNA sequences can also influence cutting and repair efficiency (Liu et al., 2016). As a result, some loci of interest may intrinsically be more difficult to target than others.

In summary, multiple CRISPR-mediated targeting approaches appear to work, but the efficiency is variable and little consensus exists, suggesting that targeting may need to be optimized for each individual locus. Here we describe our efforts to use scarless CRISPR/Cas9 mediated genome editing for scarless tagging of the Ctnnb1 gene, in order to generate a fluorescent reporter mouse that allows direct imaging of the levels and subcellular localization of the main effector of the canonical WNT signalling pathway.

Results

Optimizing and testing sgRNAs in HEK293A cells
CRISPR/Cas9 genome editing in mammalian cell lines can help to test gRNA and repair constructs prior to testing in zygotes (Jung et al., 2017). To optimize our approach, we initially sought to use a mouse cell line to compare different gRNAs and repair constructs in a tractable in vitro model system. Unfortunately, all mouse cell lines available in the lab at the time turned out to have very low transfection efficiencies, leading to insufficiently robust detection of DNA editing events. Since we were newly implementing the technology in our lab, we therefore decided to first familiarize ourselves with the promises and pitfalls of CRISPR/Cas9 genome editing in HEK293A cells. As an adherent subclone of HEK293 (Graham et al., 1977), a human cell line with high DNA transfection rates, HEK293A cells have a flat morphology that is well-suited for functional microscopy, which we aimed to exploit for image-based screening purposes.
We designed gRNAs in the vicinity of the start codon of the human CTNNB1 gene (Figure 1a) using the MIT CRISPR design tool from the Zhang lab (http://crispr.mit.edu, since retired). We selected two gRNAs closest to the CTNNB1 start codon in exon 2 with a high score (as determined by the design tool algorithm) and that were thus predicted to have a lower chance for off-target editing events (gRNA #67 and gRNA #69, Figure 1a). We designed three additional high-scoring gRNAs in exon 3 surrounding the codons encoding GSK3 and CKI phosphorylation sites (gRNA #65 for S33 and gRNA #61 and #59 for S45, Figure 1a). Finally, two high-scoring gRNAs were designed in exon 4 surrounding the codon encoding T89 (gRNA #71 and gRNA #73, Figure 1a). This collection of guide RNAs should not only allow us to perform N-terminal tagging, but also to generate a constitutively active form of CTNNB1 – either by introducing point mutations in the GSK3 and CKI phosphorylation sites (Amit et al., 2002; Liu et al., 2002), or by making larger N-terminal deletions (Munemitsu et al., 1996). To efficiently introduce both gRNA and Cas9 into the cells, the gRNA sequences were cloned into the chimeric pX330 vector, which expresses both a sgRNA and Cas9 from the same plasmid (Cong et al., 2013). All gRNA sequences are listed in supplementary table 1.

A typical workflow for testing gRNA efficiency consists of transfecting individual pX330 constructs into cells, after which their ability to generate a DSB is tested by scoring the presence of indels. Alternatively, a combination of different pX330 vectors can be transfected to introduce larger deletions, which can be scored by PCR based screening (Figure 1b,c). Indeed, we were able to generate deletions of the expected size following PCR on genomic DNA and agarose gel-based screening for different combinations of gRNAs targeting exon2 and exon 4 (Figure 1d). Different gRNA combinations appear to be more (#69 + #71) or less (#67 + #73) useful. All samples still show a band for the wildtype (WT) locus (Figure 1d, grey arrow), reflecting the fact that neither DNA transfection nor Cas9-mediated cutting are one hundred percent effective. To test the efficiency of individual gRNAs, we performed separate transfections for the three remaining gRNAs, located in exon 3 (Figure 1e). The genomic DNA isolated from these cells was tested for indels via a Surveyor assay (Qiu et al., 2004), an enzyme based assay capable of detecting small mismatches in DNA (Figure 1e). In this case, the locus of interest is amplified via PCR, then denatured and subsequently reannealed. If indels are present, this results in heteroduplexes that contain small mismatches between the DNA strands. The Surveyor enzyme recognizes these mismatches and cleaves them, resulting in smaller DNA fragments (Figure 1e, grey arrows) that are easily detected on a standard agarose gel. All three gRNAs tested introduced a sufficient number of indels to result in cleaved bands on the gel and are therefore functional. However, based on this assay alone it is difficult to determine whether one is more efficient than the other. Because multiple gRNAs were capable of introducing DSBs, either alone
An mTq2-Ctnnb1 knock-in mouse via CRISPR/Cas9 genome editing

(Figure 1e) or in combination (Figure 1d), we continued using these constructs for the next step in building our CRISPR toolbox.

Introducing small genomic changes

The real power of CRISPR/Cas genome editing lies in the possibility to create specific genomic changes, ranging from small point mutations to the seamless integration of large targeting constructs. We therefore set out to establish precision genome engineering in HEK293A. Because small modifications tend to be easier introduced than larger ones, we first decided to create point mutations in CTNNB1 at two known phosphorylation sites: S33 and S45 (Figure 1a). To do so, we designed repair templates in the form of ssODNs for each working gRNA (Supplementary table 3). These repair templates were about 100 nucleotides long, with 50bp homology arms on each side surrounding the predicted cleavage site. The repair templates contained a single base pair substitution to introduce an S33Y or S45F mutation, which are known oncogenic mutations creating a constitutively active form of CTNNB1 (Kolligs et al., 1999). Ideally, the repair template should also contain silent mutations to prevent re-cutting of the repaired sequence. The easiest way to accomplish this is by altering the PAM sequence into a non-PAM sequence (i.e. anything other than NGG or the less efficient NAG (Hsu et al., 2013; Zhang et al., 2014)). Unfortunately, this was not possible for any of the used gRNAs without altering the CTNNB1 coding sequence. Therefore, we introduced multiple silent mutations in the gRNA recognition sequence to create mismatches with the gRNA. To prevent re-cutting, there should be a minimum of three mismatches, with preferably two of these in close proximity to the PAM. Alternatively, if silent mutations cannot be introduced in the 10bp closest to the PAM site, a larger number of mismatches should be included (Hsu et al., 2013).
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Figure 1: Setting up the CRISPR toolbox: design and testing of gRNAs and introducing point mutations. a) Schematic overview of the genomic Hs CTNNB1 locus and gRNA locations. ATG: start codon of CTNNB1. S33 and S45 are known phosphorylation sites for GSK3. When mutated, they lead to a constitutive active form of CTNNB1. Deleting the first 89 amino acids also forms a constitutive active variant. b) Experimental pipeline for CRISPR/Cas9 genome editing experiments in Hek293A cells. Cells are transfected with pX330 vector containing Cas9 and gRNA sequence. 72 hours after transfection, cells are lysed and DNA is extracted and DNA sequencing is performed. c) Site-specific double stranded break with Cas9 and sgRNA for unwanted insertions/deletions. d) PCR and genome analysis with Surveyor assay or Sanger Sequencing. e) Mutant cells lane and WT lane. f) Heterozygous S33Y sequence.
extracted, followed by PCR amplification of the genomic CTNNB1 locus. This PCR product is then used as input for further analysis via Surveyor and Sanger Sequencing. c) Cartoon showing CRISPR/Cas9 genome editing to create insertions / deletions (indels) and point mutations. The Cas9/sgRNA complex binds to its gRNA recognition site followed by a PAM site (any NGG) on the genomic DNA, allowing Cas9 to create a double-stranded break. This break will be repaired via i) NHEJ, leading to indels. If ii) a single-stranded donor oligonucleotide (ssODN) containing a point mutation is introduced together with the pX330 vector, this template will serve as repair template for HDR, leading to the point mutation being introduced into the genomic locus. d) Agarose gel showing PCR products of amplified CTNNB1 following genomic DNA isolation of Hek293a cells 72 hours after transfection with Cas9 and two gRNAs to create a deletion and wildtype control. Expected wildtype band (grey arrow) is at 1222 bp, deletion depending on gRNAs ~850-1000bp (black arrow). Note that in all lanes, an a-specific ~200bp band is found in all samples (Asterix). e) Surveyor Assay showing indels in a pool of cells transfected with Cas9/gRNAs to check for sgRNA efficiency. Surveyor Nuclease cuts mismatches of re-annealed DNA heteroduplexes amplified from cells, leading to smaller DNA fragments (grey arrows) than the homoduplex WT band (dark arrow). f) Sequence read showing the CTNNB1 sequence of a Hek293a clonal cell line with a heterozygous point mutation (green arrow) leading to S33Y, which is a constitutively active CTNNB1 variant.

HEK293A cells were transfected with individual pX330 constructs together with the appropriate repair template (Supplementary Table 3). Three days after transfection, some of the cells were lysed to isolate the genomic DNA, which was analysed by Surveyor assay as described above (data not shown). All conditions resulted in some cleaved products on gel, but based on a Surveyor assay alone it is impossible to discriminate between point mutations (i.e. cutting and successful HDR-mediated repair) and indels (i.e. cutting followed by NHEJ). Therefore, the remaining cells were used to generate clonal cell populations via limiting dilution. DNA was isolated from these clones to check the CTNNB1 locus by Sanger sequencing. For S33Y, six clones were analysed, of which one was heterozygous for the designed mutation (Figure 1f). The remaining five clones all had wildtype CTNNB1 alleles. For S45F, seven clones were analysed. Of these seven, one clone showed a homozygous point mutation (data not shown). Of the remaining five clones, four were wildtype and one showed indels but no repair. It is not clear whether the wildtype clones were not successfully transfected to begin with, or whether CRISPR editing did not happen.

Together, these results suggest that the introduction of point mutations via ssDNA repair templates is quite efficient, especially considering the absence of any selection procedure, with 2/13 tested clones having the desired mutation. This corresponds to a 14% (1/7, S45F) to 17% (1/6, S33Y) success rate for the generation of both homozygous and heterozygous mutations.
Generating scarless fluorescent fusions in the endogenous CTNNB1 locus

To test if larger genomic fragments could be successfully targeted to the CTNNB1 locus as well, we next set out to generate a scarless knock-in of several fluorescent protein (FP) encoding genes (717bp), designed to be expressed in frame with the endogenous CTNNB1 protein. Here, we considered multiple design criteria.

CTNNB1 is highly conserved between species (781 amino acids with only a single mismatch between mouse and human), with a predicted molecular mass of ~85 kDa. It contains a central region with 12 so-called armadillo repeats, flanked by more flexible N- and C-terminal regions. The armadillo repeats form a solid core with a groove that facilitates interactions with the many CTNNB1 binding partners, such as APC, CDH1 and TCF/LEF (Valenta et al., 2012). Attaching a fluorescent protein could block binding of these partners, so it should not be inserted in the armadillo repeats. This leaves the N- and C-terminus as candidate sites for tagging CTNNB1. Fusions at the FP N-terminus (i.e. C-terminal fusions of the protein of interest) often require a short linker sequence to create space so that the FP again does not inhibit any biological functions of its fusion partner by steric hindrance (Joachim Goedhart, personal communication). In contrast, the C-terminus of GFP-based FPs sticks out and therefore acts as a linker itself when inserted at the N-terminus of the protein of interest, thereby eliminating the need to introduce anything else than just the FP sequence. Together with the fact that N-terminal CTNNB1 fusions had previously been reported to be functional (Murase et al., 2002), we therefore decided to create a scarless N-terminal FP-CTNNB1 fusion, where the FP would be inserted precisely at the start-codon of CTNNB1. Importantly, this means that all regulatory, non-coding sequences of CTNNB1 such as the promoter, enhancer elements and UTRs should not be affected, and the targeted allele should behave as wildtype.

When creating an endogenous fluorescent fusion protein, it is important that the FP used is monomeric. This prevents multimerization of the fluorescent fusion protein, which could otherwise lead to non-functional aggregates and mislocalization of the protein under study. Also, it should be bright and ideally facilitate detection of single molecules at low endogenous concentrations for quantitative imaging analyses. Finally, the ideal FP would have high photostability so that it does not bleach during prolonged measurements, for example when creating Z-stacks in wholemount tissues or when performing live-cell imaging over time. Based on these criteria, we selected four different bright monomeric FPs to test their performance in functional imaging at endogenous expression levels: mTurquoise2 (mTq2) (Goedhart et al., 2012), SGFP2 (Kremers et al., 2007), SYFP2 (Kremers et al., 2006) and mCherry (Shaner et al., 2004). Together they cover the most commonly used channels in fluorescence microscopy (cyan, green, yellow and red).
Figure 2: Creating a tagged FP-CTNNB1 cell line. a) Experimental pipeline for tagging CTNNB1 in Hek293a cells. Cells are transfected with pX330 vector containing Cas9, gRNA sequence and repair plasmid containing homology sequences and the desired fluorescent protein sequence. 72 hours after transfection, cells are directly imaged at a confocal microscope. b) Cartoon showing the tagging strategy to generate an FP-CTNNB1 reporter cell line in Hek293a cells. The locus is opened by directing Cas9 to the closest possible gRNA site to the ATG of CTNNB1. A double stranded repair plasmid containing a fluorescent protein (FP) with 800bp homologous regions (HR) is introduced into the cells simultaneously to induce homology directed repair. The repair template is designed to insert the FP at the ATG of CTNNB1 in a scarless manner, resulting in fusion protein. c) Confocal images of Hek293a cells fluorescently tagged at the endogenous CTNNB1 locus using CRISPR/Cas9. Cells shown are from FACS enriched populations and tagged with four different fluorescent proteins: SYFP2, SGFP2, mCherry and mTq2. d) Hek293a cells tagged with mTq2 at beta-catenin treated with the GSK3-inhibitor CHIR show an increase in the fluorescent signal.
We generated circular repair plasmids containing the coding sequence for each of these FPs, flanked by 800bp CTNNB1 homology arms on either side (Figure 2a, 2b). We transfected these plasmids together with what appeared to be the most efficient gRNA in the vicinity of the start codon (#69, Figure 1d). To determine if editing had been successful, we took advantage of the fact that we could now perform confocal microscopy based, rather than PCR and sequencing-based screening (Figure 2a).

Because the FP-CTNNB1 is designed as a fusion protein, the FP should localise to the same subcellular locations as CTNNB1. Indeed, at 72 hours after transfection for each of the four different FPs we could detect rare patches of cells where the FP appeared to be successfully inserted (Figure 2c). As expected, FP-CTNNB1 localizes predominantly at punctate structures at the cell membrane, which likely represent adherens junctions (Jamora and Fuchs, 2002; Yamada et al., 2005). WNT signalling is not stimulated in HEK293A cells, so no fluorescence can be detected in the nucleus (Behrens et al., 1996). However, very low levels of fluorescence could be measured in the cytoplasm, as expected from the low endogenous levels of CTNNB1 found when the destruction complex is active (Tan et al., 2012).

To test if the fluorescent signal actually reported FP-CTNNB1 and not a random FP integration event, we activated WNT/CTNNB1 signalling by treating the mTq2-CTNNB1 cell pool with 3µM of the GSK3-inhibiter CHIR99021 (Ring et al., 2003) for 24 hours prior to imaging. In some cells, GSK3 inhibition indeed resulted in an increase in fluorescence, indicating that in those cases the signal originated from successful targeting events (Figure 2d). Overall, mCherry-CTNNB1 was the most difficult to visualize and SYFP2-CTNNB1 suffered the most from bleaching as a result of the high laser power needed to detect the endogenous signal. Although feasible, microscopy-based screening was challenging and time-consuming, due to the low endogenous fluorescent signal and inefficient tagging. The latter also made it highly impractical to derive clonal cell populations by limiting dilution. We therefore turned to fluorescence activated cell sorting (FACS) to determine if we could isolate successfully tagged cells in a more high-throughput fashion (Figure 3a).

Fluorescence-activated cell sorting (FACS) of CRISPR/Cas9 treated cells transfected with SGFP2 or mTq2 repair plasmids confirmed that only a small proportion of cells was FP-positive, albeit barely above background (Figure 3b). Here too, treatment with CHIR99021 resulted in a clear increase in the fluorescence intensity signal (data not shown). Sorting and re-analysing cells with the highest fluorescence intensity signal (top 0.5-2%) showed that we could indeed enrich for FP-positive cells, although for mTq2-CTNNB1 the signal remained difficult to distinguish from background (Figure 3c). The FACS settings and laser lines available for the blue channel do not match the optimal excitation and emission wavelengths for detecting the cyan fluorescent protein mTq2 (Figure 3d). In addition, the blue part of the spectrum induces relatively more autofluorescence then the red-shifted
An mTq2-Ctnnb1 knock-in mouse via CRISPR/Cas9 genome editing

part (Andersson et al., 1998; Aubin, 1979), which can be especially challenging when dealing with low-level fluorescence.

From the enriched cell populations, clonal cell lines could be derived by limiting dilution for both SGFP2-CTNNB1 and mTq2-CTNNB1 (Figure 3e). Three of these lines were further analysed by PCR and Sanger sequencing of the region encompassing the CTNNB1 start codon. None of the clones were homozygously tagged, as revealed by the presence of a PCR product with the predicted size of the wildtype allele (data not shown). Furthermore, multiple sequence traces were present surrounding the cut site, revealing the presence of more than two different alleles per clone (data not shown). Indeed, HEK293A cells are hypotriploid.

Tagging CTNNB1 in a mouse mammary epithelial cell line

Having optimized the different steps of our scarless CRISPR/Cas9 mediated tagging in HEK293A cells, we returned to a mouse cell line. We selected the mouse mammary epithelial cell line BC44 (Deugnier et al., 1999). We did so for a dual purpose: First, it would allow further testing and optimization of our workflow and gRNA testing in vitro before attempting to perform scarless tagging in mouse zygotes. Second, due to its known WNT-responsiveness (Bresson et al., 2018), generating a BC44 mTq2-Ctnnb1 cell line would be a useful tool to study the role of WNT/CTNNB1 signalling in mammary gland biology.

We designed gRNAs for mouse Ctnnb1 in a similar way as for the human locus. Four new gRNAs were designed surrounding the ATG (Figure 4a and Supplementary table 1: gRNA mmCTNNB1 1-4) and tested for their ability to create indels. Based on our prior experience, we made some modifications to our workflow: because BC44 cells are less efficiently transfected than HEK293A cells, the gRNAs were cloned into the pX462 expression vector, which is similar to pX330 except for an additional puromycin selection cassette to select and enrich for successfully transfected cells (Ran et al., 2013a). Furthermore, instead of using the enzyme-based Surveyor assay to score gRNA efficiency, DNA isolated from transfected BC44 cells was analysed for homo- and heteroduplexes directly by running PCR products on a non-denaturing polyacrylamide gel (PAGE) (Chen et al., 2012; Sambrook and Russell, 2001) (Figure 4b). Heteroduplexes containing mismatches between the DNA strands run slower than homoduplexes without mismatches. As expected, a strong single band of homoduplexes was found in cells transfected with an empty pX462 vector (arrowhead), while cell populations transfected with pX462 containing one of the four gRNAs in addition showed a smear of heteroduplex bands (triangle).
Chapter 6

1) Transfection

\[
\text{pX330 vector} + \text{Repair plasmid}
\]

2) Sorting by FACS followed by live cell imaging

72 hours

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a) mTq2-CTNNB1

\[
\begin{array}{c}
\text{SGFP2-CTNNB1} \\
\text{mTq2-CTNNB1}
\end{array}
\]

<table>
<thead>
<tr>
<th>Count</th>
<th>GFP-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>120K</td>
<td>90K</td>
</tr>
<tr>
<td>90K</td>
<td>60K</td>
</tr>
<tr>
<td>60K</td>
<td>30K</td>
</tr>
<tr>
<td>30K</td>
<td>0</td>
</tr>
</tbody>
</table>

mTq2 positive 0.85%

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b) 470/40 filter

\[
\begin{array}{c}
\text{DAPI-A} \\
\text{DAPI-A}
\end{array}
\]

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c) 540/50 filter

\[
\begin{array}{c}
\text{DAPI-A} \\
\text{DAPI-A}
\end{array}
\]

---

d) SGFP2-CTNNB1

\[
\begin{array}{c}
\text{pre-sort} \\
\text{post-sort}
\end{array}
\]

\[
\begin{array}{c}
\text{GFP-A} \\
\text{DAPI-A}
\end{array}
\]

---

e) mTq2-CTNNB1

\[
\begin{array}{c}
\text{SGFP2-CTNNB1} \\
\text{mTq2-CTNNB1}
\end{array}
\]

\[
\begin{array}{c}
\text{pre-sort} \\
\text{post-sort}
\end{array}
\]

\[
\begin{array}{c}
\text{GFP-A} \\
\text{DAPI-A}
\end{array}
\]

---

Normalized absorbance (-)

Wavelength (nm)

500

540

510

550

600

650

-
Figure 3: Enriching for FP-CTNNB1 tagged cells via FACS sorting. a) Experimental pipeline for creating FP-CTNNB1 Hek293a cells. Cells are transfected with pX330 vector containing Cas9, gRNA sequence and repair plasmid containing homology sequences and the desired fluorescent protein sequence. 72 hours after transfection, cells are FACS sorted to enrich for fluorescent cells. b) Graphs showing cell count for depicted fluorescent channels to detect FP-CTNNB1 cells. Left: GFP-A channel showing a high peak for wildtype cells, and nearly no events (1.9%) at higher fluorescence levels (dashed line), where SGFP2-CTNNB1 positive cells are expected. Right: DAPI-A channel with 470/40 and 510/50 emission filters showing a high peak for wildtype cells, and no visible cell count (events falling in gate: 0.56% and 0.49%) at higher fluorescence (dashed line), where mTq2-CTNNB1 positive cells are expected. c) Graph showing fluorescent signal in a pool of cells transfected with Cas9/gRNA/FP repair plasmid pre-sorting and post sorting. On average, the post-sort population for SGFP2 displays a higher SGFP2 signal, while the post-sort population for mTq2 only has a small shoulder displaying higher mTq2 fluorescence compared to the pre-sort population. d) Graph showing excitation and emission spectre for mTq2. A 407 laser is used, that is that is suboptimal for exciting mTq2. Detection channels 470/40 and 510/50 were used to identify mTq2 positive cells. Both only use part of the emission spectrum and are therefore suboptimal. e) Confocal image showing a clonal Hek293a cell line tagged with mTq2 at the start codon of CTNNB1.
Chapter 6

1) Transfection
   pX462 vector + Repair plasmid
   72 hours

2) FACS sorting
   Recover

3-i) Limiting dilutions
   or
   3-ii) Re-sorting

a. Mm-Ctnnb1

b. WT Pool of mutant cells

gRNA none 1 2 3

SD 5.3 2.5 5.6

c. gRNA efficiency with TIDE tool

8.1% 18.1% 7.9%

d. Exon 2

-9 bp 0 8 bp

1 start 3

e. Transfected cells

Enriched population

Clonal cell lines

f. mTq2-CTNNB1

CTNNB1

Tubulin

WT Clones

1 2 3 4 5 6 7 8 9 10 11

Axin2

Fold change

Procr

Fold change
Figure 4: generation of mTq2-CTNNB1 clonal lines in mouse mammary gland cells. a) Schematic overview of the genomic mm Ctnnb1 locus and gRNA locations surrounding the start codon. b) PAGE gel showing indels in a pool of cells transfected with Cas9/gRNAs to check for sgRNA efficiency. DNA homoduplexes (arrowhead) run quicker than DNA heteroduplexes (accolade) containing DNA mismatches due to indels. c) TIDE tool analysis showing calculated gRNA efficiencies and standard deviation for the gRNAs depicted on the PAGE gel. N=3 NA: primers used were too far from the gRNA 4 site to calculate a reliable efficiency. d) Strategy to obtain clonal cell lines. WT BC44 cells are transfected with CRISPR/repair constructs and an enriched population is obtained by FACS. This enriched population is used as input for single-cell FACS or limiting dilutions to obtain clonal populations derived from single cells. Shown in the 3rd panel is “clone 1”. e) Confocal images showing examples of BC44 cells transfected with CRISPR/repair constructs (left), an enriched population obtained via FACS (middle) and a clonal cell line with all cells showing mTq2-CTNNB1 signal (right). f) Western Blot showing mTq2-CTNNB1 (115 kDa) and wildtype CTNNB1 (88 kDa) in 11 clonal lines. Some lines are homozygous for mTq2-CTNNB1 (clone 6, 7, 8, 10, 11), 5 others do not (clone 1, 2, 4, 5, 9). Clone 3 is scored as ND (non-determined). Tubulin (55 kDa) was used as a loading control. g) Graph showing qPCR results for expression of Wnt target genes Axin2 and Procr for a pool of WT BC44 cells, compared to clone 1 and 2 as treated with DMSO or 3µM CHIR for 4 and 24 hours. N=1, error bars showing SD for technical replicates. Rpl13a and Gapdh were used as reference genes, a geometric mean was determined for both reference genes to normalise samples.

Although PAGE analysis is a relatively straightforward way to determine if the gRNAs work, like the Surveyor assay, it does not reveal how efficient they are. To determine the amount of indels in a more quantitative manner, we therefore analysed the samples via the Tracking of Indels by Decomposition (TIDE) tool (Brinkman et al., 2014). Following PCR and DNA sequence analysis, this tool compares Sanger sequencing data from a pool of cells transfected with Cas9/gRNA to an unedited wildtype sequence and extrapolates this into a score for gRNA efficiency and an overview of the most prominent indels. This method is easy and relatively cheap, especially compared to the enzyme-based Surveyor method. However, the purity of the PCR and the quality of the sequencing must be high in order to get reliable results. To analyse indel efficiency, a primer set is needed that amplifies a ~700bp stretch of DNA of the targeting locus. The projected cut site should ideally be located ~200bp downstream from the sequencing start site.

The gRNAs tested by PAGE analysis (Figure 4b) were also analysed using the TIDE tool following PCR and Sanger sequencing (Figure 4c). gRNA 1, 2 and 3 are located close together and therefore can be tested with the same set of primers. gRNA 4 was designed later and was therefore not tested together with this set. Based on the results from the TIDE analysis, gRNA 2 is most efficient (Figure 4c), whereas gRNA 3 shows the most prominent heteroduplex formation (Figure 4b). The PAGE analysis
was only performed once. While the TIDE analysis was performed three times, it showed variation over the separate analyses from three different PCR reactions. Taken together, the Surveyor and PAGE assay are quick options to check for cutting activity of gRNAs, but TIDE can provide more detail on the cutting efficiency and indel size frequencies.

Because all three gRNAs showed some activity (Figure 4b) and TIDE analysis did not suggest large differences in cutting efficiency (Figure 4c), we continued with gRNA 1 and 3, because their predicted cut-site is closer to the ATG, respectively 16 and 6 basepairs (instead of 23 for gRNA 2). Using our optimized workflow, we sought to tag endogenous CTNNB1 in BC44 cells with mTq2 (Figure 4d). Following transfection, we detected few cells with the expected membrane-localized fluorescence expression by manual screening using confocal microscopy (Figure 4e, left). FACS sorting allowed the isolation of an enriched mTq2-positive population (Figure 4e, middle). Therefore, FACS was used to create an enriched population of fluorescent cells. To create clonal cell lines from this enriched population, two methods were compared: setting up limiting dilutions and FACS-based re-sorting of single cells. The former resulted in 5 clones, with 3/5 (60%) showing the expected localization of mTq2-CTNNB1 and 2/5 (40%) not showing any fluorescent signal at all when analysed by confocal microscopy. Following FACS sorting of the enriched population into single cells, 33/96 clones grew out. Of these, 29/33 (88%) were positive for the fluorescent mTq2-CTNNB1 signal and 4/33 (12%) were negative. Of note, all of these clones resulted from one single transfection experiment with gRNA 1 and they can therefore not be considered to be independent.

Eleven clones with the expected localization of mTq2-CTNNB1 (Figure 4e, right) were analysed in further detail. To check if the fusion protein ran at the expected height, a Western Blot was probed with an anti-CTNNB1 antibody (Figure 4f). Tagged mTq2-CTNNB1 is expected to run higher than the wildtype protein, at approximately 115 kDa. Ten clones (1-2 and 4-11) indeed showed a band of the expected size for mTq2-CTNNB1, with 5/10 still showing a wildtype band as well, indicating that they were heterozygously tagged. Thus, 5/10 (50%) clones appear to be homozygous knock-ins. Following the same workflow, clonal cell lines were also generated for SGFP2-CTNNB1. Of twelve positive clones analysed via confocal microscopy and Western Blot, none were homozygously tagged (data not shown).

We selected two homozygous mTq2-CTNNB1 clones for further analysis. Sequencing of the locus showed correct scarless integration of mTq2 in the Ctnnb1 locus as designed (data not shown). To check if the Wnt signalling response in these clones was still intact after the tagging and clonal selection process, the induction of two known Wnt target genes was analysed by qRT-PCR after stimulation with 3µM of the Wnt agonist CHIR99021, and compared to the response of a pool of wildtype BC44 cells (Figure 4g). The Wnt response between the wildtype pool and clones differed substantially. Clone 1 retained Wnt responsiveness based on Axin2, but not
Procr induction. Clone 2, however, appeared to have lost Wnt responsiveness altogether: upon addition of CHIR99021, the expression of neither Axin2, nor Procr increased. At present we have no explanation for this observation. However, when we performed immunofluorescence staining for the keratin marker K14, which is normally expressed in BC44 cells (Deugnier et al., 1999), we observed that Clone 2 cells were also no longer K14+. Thus, we discontinued experiments with these BC44 cell lines.

**Generation of an mTq2-CTNNB1 knock-in mouse**

Having validated our guide and repair constructs for tagging murine CTNNB1 in vitro, we used these same constructs for injection into fertilized FVB/N oocytes (Figure 5a,b). For in vivo gene editing purposes, Cas9 and gRNA can be supplied as in vitro translated RNAs (Yang et al., 2013) or as a pre-assembled ribonucleoprotein complex (Quadros et al., 2017). When we first began these experiments, CRISPR/Cas9-mediated gene-editing in vivo was very much still in the pioneering stage. Therefore, we empirically determined the conditions that would allow the efficient and scarless generation of mTq2-Ctnnb1 knock-in mice – something that requires both a sufficiently high rate of embryo survival and on-target integration of the knock-in construct. In total, we tested 13 different conditions, including four different repair constructs (figure 5c), over a period of 25 months (listed in Table 1 as attempts 1-13).

We started with an approach based on a published protocol (Yang et al., 2014), which uses an injection mix of RNA (Cas9 and gRNA gene editing components) and circular plasmid DNA (repair template). During our first targeting attempt, all zygotes died. Upon scrutinizing our workflow and the literature, we realized that this was most likely due to the fact that we performed pronuclear, rather than cytoplasmic injections. Henceforward, we reduced the concentrations and total amount for each of the components in the injection mix, which solved the issue of toxicity.
### Table 1. Overview of zygote injections to knock-in a gene encoding mTurquoise2 into

<table>
<thead>
<tr>
<th>attempt</th>
<th>DNA repair template</th>
<th>Cas9</th>
<th>gRNA</th>
<th>injection mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>800 + 800 bp RNA gRNA 1</td>
<td>gRNA 50 ng/µl; Cas9 100 ng/µl Repair 200 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>800 + 800 bp RNA gRNA 1</td>
<td>gRNA 25 ng/µl; Cas9 50 ng/µl Repair 2 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>800 + 800 bp RNA gRNA 1</td>
<td>gRNA 25 ng/µl; Cas9 50 ng/µl Repair 10 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>800 + 800 bp RNA gRNA 1</td>
<td>gRNA 25 ng/µl; Cas9 50 ng/µl Repair 50 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>800 + 800 bp RNA gRNA 1</td>
<td>gRNA 50 ng/µl; Cas9 100 ng/µl Repair 100 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2000 + 2000 bp RNA gRNA 1</td>
<td>gRNA 25 ng/µl; Cas9 50 ng/µl Repair 10 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2000 + 2000 bp RNA gRNA 1</td>
<td>gRNA 25 ng/µl; Cas9 50 ng/µl Repair 10 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>120+60 bp RNA gRNA 1</td>
<td>gRNA 25ng/µl; Cas9 50ng/µl; lssDNA 5 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>300+60 bp RNA gRNA 1</td>
<td>gRNA 25ng/µl; Cas9 50ng/µl; lssDNA 5 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>120+60 bp RNA gRNA 1</td>
<td>gRNA 25ng/µl; Cas9 50ng/µl; lssDNA 5 ng/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>120+60 bp protein gRNA 5</td>
<td>gRNA 25ng/µl; Cas9 prot 200 ng/µl; LssDNA 20 ng/µl</td>
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<tr>
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<td>gRNA 10ng/µl; Cas9 prot 20 ng/µl; LssDNA 10 ng/µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Includes serine linker so the gRNA cannot recut the repaired locus.
2 Newly designed gRNA according to novel design rules
3 Mix was toxic upon pro-nuclear injection; amounts are meant for cytoplasmic injection.
An mTq2-Ctnnb1 knock-in mouse via CRISPR/Cas9 genome editing

the mouse CTNNB1 locus

<table>
<thead>
<tr>
<th>injected</th>
<th>cleaved to 2-cell (%)</th>
<th>developed to blastocyst stage</th>
<th>survival (% of injected)</th>
<th>analysed by PCR</th>
<th>mTq2 integration</th>
<th>targeting success (% of analysed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>0 (0%)</td>
<td>-</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>96</td>
<td>67 (70%)</td>
<td>n.d.</td>
<td>96</td>
<td>23 (24%)</td>
<td>0</td>
<td>0/23 (0%)</td>
</tr>
<tr>
<td>132</td>
<td>71 (54%)</td>
<td>38 (29%)</td>
<td>132</td>
<td>37 (29%)</td>
<td>1 (5%)</td>
<td>0/37 (0%)</td>
</tr>
<tr>
<td>46</td>
<td>12 (26%)</td>
<td>7 (15%)</td>
<td>46</td>
<td>5 (15%)</td>
<td>0</td>
<td>0/5 (0%)</td>
</tr>
<tr>
<td>90</td>
<td>10 (11%)</td>
<td>1 (1%)</td>
<td>90</td>
<td>1 (1%)</td>
<td>0</td>
<td>0/1 (0%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>65</td>
<td>n.d.</td>
<td>60</td>
<td>0</td>
<td>1/60 (1.7%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>132</td>
<td>20 (26%)</td>
<td>2 (5%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>8</td>
<td>46</td>
<td>8</td>
<td>0</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>33</td>
<td>33</td>
<td>1 (5%)</td>
<td>0</td>
<td>0/33 (0%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>32</td>
<td>32/50 (64%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>23</td>
<td>23/46 (50%)</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>24</td>
<td>24</td>
<td>23</td>
<td>3</td>
<td>3/23 (13%)</td>
</tr>
</tbody>
</table>
Figure 5: Experimental pipeline for the generation of mTq2-CTNNB1 mice. a) Diagram showing the generation of mTq2-CTNNB1 mice from zygotes. Zygotes are injected with Cas9, gRNA and repair construct and monitored for development into blastocysts. For quick analysis of successful tagging, blastocysts are genotyped directly. To create mice, blastocysts are transferred into pseudo-pregnant females. In case of successful tagging, offspring (F0) is often mosaic and should be checked for germline transmission of the targeted allele. b) Schematic representation of the mouse Ctnnb1 locus (chr9:120929216-120960507, mm10 coordinates). Exons depicted as boxes (white = UTR, grey = CDS). c) Schematic representation of repair constructs used to target the Ctnnb1 locus with mTq2 at the start codon in exon 2. 800bp and 2kb homology arms were introduced as plasmid DNA (top). 300 + 60 bp and 120 + 60 bp homology arms were introduced as single-stranded DNA oligos (bottom). d) Schematic overview of primer locations for genotyping wildtype and knock-in locus (left). FW: forward primer. RV: reverse primer. Agarose gel showing PCR bands for wildtype (379bp) or knock-in (324bp) in heterozygous (HET), wildtype (WT) or homozygous (HOM) mTq2-CTNNB1 mice.
A high concentration of Cas9/gRNA/repair template can lead to higher editing success in the zygote, but also increases the chance of retarded development (Yang et al., 2013). Most likely, this is due to an increased risk of toxicity or higher rates of off-target events (Kimberland et al., 2018). Indeed, we observed a clear correlation between the amount of repair plasmid that was injected and survival of the embryo’s (Table 1, attempts 2-5): The percentage of embryos that cleaved to the 2-cell stage ranged from 11%-70%. Of these 2-cell embryos, 10%-54% successfully developed into blastocysts, the point at which they are implanted into pseudo-pregnant fosters (Figure 5a). We initially aimed at analysing our targeting success by directly screening mice (Table 1, attempt 2), but this meant having to wait ~3-4 weeks before results could be obtained. Therefore, we switched our approach to PCR-based screening of blastocysts prior to implantation, so that analysis could be performed only a few days after each injection round.

To gain insight into the targeting process at the DNA level, all samples showing large deletions or integrations after PCR-based amplification of the region of interest were analysed by Sanger sequencing. Small indels, which are not detected on agarose gel electrophoresis, were not investigated further. A schematic overview of the genetic changes found upon targeting with gRNA #1 and a repair plasmid with 800bp homology arms (Table 1, attempts 3-5) is depicted in Figure 6a. In a total of 29 blastocysts for which DNA sequence information was obtained, no correct mTq2 integrations occurred. However, several large deletions were found (Figure 6a, del 1 – del 3), including homozygous ones (Figure 6a, del 1), suggesting that gRNA #1 was cutting efficiently. One partial integration of a 5’ portion of mTq2 was found (Figure 6a, part int), revealing a promising, but futile attempt at HDR from the circular DNA repair template. Finally, two samples showed evidence of small deletions in combination with independent random integrations of a related genomic region (Figure 6a, r int 1 & 2). A BLAST search for these integrated DNA sequences (red box in Figure 6a) returned hits all over the mouse genome. The random integrations of 350 and 450bp thus represent a repetitive sequence with sequence homology to an endogenous retroviral element (ERV), specifically: Erv3, of which approximately 100 copies exist, distributed across the mouse genome. A perfect match was detected with an Erv3 sequence on chromosome 1. Thus, random sequences can integrate at the site of the double-strand break and some sequences (such as the Erv3 sequence highlighted in red in Figure 6a) may be more prone to do so than others. Since ERVs are known to display retrotransposon activity, this particular insertion may have caught such a retrotransposon event in the act, allowing it to integrate at the site of the CRISPR induced double strand break. A similar observation was recently reported for other target sites, indicating that the insertion of retroelements in early embryos, when these elements are activated as a result of global demethylation of the genome, may be a common occurrence (Jeon et al., 2019).
Figure 6: Overview of large genetic changes found in blastocysts or mice after CRISPR injections. a) Ctnnb1 locus with 800bp homology arms (green, top row), and designed locus after repair with mTq2 knock-in (blue, 2nd row). Depicted underneath are all large genetic changes found from a total of 43 screened blastocysts (attempts 3-5 as depicted in table 1). 3 Deletions (del) were found, one partial integration (part int) of mTq2, and two random genomic regions were integrated (r int). The red box in r int 1 and 2 is part of the same DNA stretch (matching Erv3), but 2 is in the reverse orientation compared to 1. The purple box is part of another random DNA stretch. Zygotes were injected with Cas9 mRNA, gRNA 1 and plasmid with 800bp homology arms. b) Ctnnb1 locus with 2kb homology arms (green, top row), and designed locus after repair with mTq2 knock-in (blue, 2nd row). Depicted underneath are large genetic changes found from a total of 60 screened blastocysts (attempt 6 as depicted in table 1). One correct integration (int) was found and 2 deletions (del). Zygotes were injected with Cas9 mRNA, gRNA 1 and designed for gRNA 3. c) Ctnnb1 locus with 120/60bp homology arms (green, top row), and designed locus for gRNA 1 after repair with mTq2 knock-in (blue, 2nd row). Depicted underneath are large genetic changes found from a total of 28 screened mice (attempt 8 and 9 as depicted in table 1). Two partial mTq2 integrations (part int) were found and one of those sample also carried a random integration of a part of the gene Nfs1. No other large genomic changes were detected. Zygotes were injected with Cas9 mRNA, gRNA 1 and long single-stranded oligos with 120/60bp arms. d)
An mTq2-Ctnnb1 knock-in mouse via CRISPR/Cas9 genome editing

Designed locus for gRNA 3 after repair with mTq2 knock-in, including a Serine linker (yellow) to prevent re-cutting of the gRNA. Depicted underneath are large genetic changes found from a total of 33 screened mice (attempt 10 as depicted in table 1). One partial mTq2 integration (part int) was found, no other large genomic changes were detected. Zygotes were injected with Cas9 mRNA, gRNA 3 and long single-stranded oligos with 120/60bp arms.

In an attempt to increase repair efficiency, the length of the homology arms was increased to 2kb. This did lead to a correct knock-in of mTq2 at the Ctnnb1 locus in 1 out of 60 blastocysts analysed (Table 1, attempt 6). The sequence of this integration was completely as designed, as verified by Sanger sequencing (Figure 6b). No random integration events were detected in this targeting attempt, but some large deletions, spanning more than 1kb, were found (Figure 6b). Even though this attempt resulted in our first successful knock-in, we deemed a success rate of 1.7% to be too low to efficiently create novel knock-in mouse lines. We therefore sought to further improve our strategy. Of note, the use of another guide in combination with the same repair plasmid did not increase the KI targeting rate (gRNA #3, Table 1, attempt 7).

Around this time, the use of long single stranded (Lss) oligos was reported with promising results (Quadros et al., 2017), so this was tried as an eighth and ninth attempt, using two different lengths of asymmetric homology arms (120+60 and 300+60bp) in combination with gRNA #1 (Table 1, attempt 8 and 9). Again, this did not result in a correct knock-in of mTq2. However, two partial integrations were found when using an Lss DNA oligo with 120+60bp arms (Figure 6c). In the first case, the first 339 bases of mTurquoise2 were properly knocked in on the 5’ end, but 3’ recombination was not executed successfully (Figure 6c, part int 1). In the second case, the first 552 bases of mTq2 were knocked in as intended on the 5’ end, but a non-specific sequence had been co-inserted on the 3’ end (Figure 6c, part int 2). DNA sequencing and BLAST analysis revealed that the non-specific sequence showed perfect homology to a 472bp stretch of the 3’ UTR of the Nfs1 (nitrogen fixation gene 1) gene. Unlike the inserted ERV3 sequences, this DNA sequence does not show any signs of harbouring repetitive elements or having a retroviral origin. It also does not show any obvious homology to either the repair template or the targeting site. Moreover, it is not derived from the same chromosome: Nfs1 is located on chromosome 2, while Ctnnb1 is located on chromosome 9.

Upon re-assessing our gRNAs with novel scoring algorithms, such as the CRISPOR tool available via http://crispor.tefor.net (Haeussler et al., 2016), we decided to switch to a different gRNA (gRNA #3), which we had already tested earlier for its capacity to induce DSB in exon 2 of Ctnnb1 (Figure 4b,c). Because gRNA #3 cuts slightly further upstream from the desired integration site (i.e. the Ctnnb1 start codon) than gRNA #1, following repair too much of the gRNA recognition site remains intact. This can lead to re-cutting and the generation of indels even after
successful targeting of the locus. To prevent such disruption of the newly generated knock-in allele, we therefore modified the Lss DNA oligo repair template by introducing a Serine linker between the last coding exon of mTq2 and the start codon of Ctnnb1 (Table 1, attempt 10). Although 1 out of 33 mice analysed showed a 115bp partial integration of mTq2 on the 5’ end of the targeting site, no successful knock-ins were detected (Figure 6d).

Finally, we decided to re-assess all potential gRNA sequences with newly developed CRISPR tools and design algorithms to see if we could design a new gRNA that would cut within 8bp of the predicted cut and integration site and that would allow scarless editing. One gRNA that was originally discarded because of a low score in the MIT CRISPR design tool (Hsu et al., 2013), with an estimated cut site 4bp downstream of the 5’ homology arm, received a high score from other CRISPR prediction algorithms, including the online CRISPOR tool (Haeussler et al., 2016). Each of those predicted it to have a small chance for off-target cutting. We also changed a second variable, by introducing Cas9 as in vitro translated protein instead of mRNA. When introducing Cas9 protein in a pre-assembled Cas9/sgRNA ribonucleoprotein particle (RNP) in combination with Lss oligos, very high (up to 67%) success rates of creating knock-in mice had been reported (Chen et al., 2016; Quadros et al., 2017). Indeed, the combination of Cas9 protein and our new Lss DNA oligo turned out very efficient: over half (55/96) of all analysed blastocysts were correctly tagged as designed (Table 1, attempts 11 and 12). Two different gRNA/Cas9/repair concentrations were used for the blastocysts (Miura et al., 2018). The higher concentrations lead to slightly higher loss of embryos at the blastocyst stage, but those surviving were more often successfully tagged. Partial mTq2 integrations, random integrations and large deletions were not detected in these injection rounds. We repeated this experiment, but this time analysed mice instead of blastocysts. Of 23 pups born, 3 (13%) had a correct mTq2-Ctnnb1 knock-in (Table 1, attempt 13).

**Characterization of mTq2-Ctnnb1 mice**

Germline transmission of the targeted allele was successful and a colony of heterozygous mTq2-Ctnnb1 knock-in mice was established on an inbred background by backcrossing to FVB/N. Mice homozygous for the mTq2-Ctnnb1 knock-in allele were born from heterozygous intercrosses (Figure 5d). They were viable and fertile, without any apparent phenotype. Because compromised WNT/CTNNB1 signalling results in more or less severe developmental phenotypes (van Amerongen and Berns, 2006), this suggests that the expression and function of our N-terminally tagged Ctnnb1 allele is not compromised.
An mTq2-Ctnnb1 knock-in mouse via CRISPR/Cas9 genome editing

Figure 7: Generation and detection of mTq2-CTNNB1 in mice. a) Diagram showing the generation of mTq2-CTNNB1 mice from zygotes. Zygotes are injected with 20ng/µl Cas9 protein, 10ng/µl sgRNA and 10ng/µl LSS oligo. and monitored to develop into blastocysts. They are transferred into pseudo-pregnant females to further develop. Offspring (F0) was checked for germline transmission. b) Microscopy images showing sections of the intestine of wildtype balb/c mice with immunohistochemistry labelling for CTNNB1 and counterstained with hematoxylin. CTNNB1 is found at the adherens junctions between cells. Arrowheads show nuclear accumulation of CTNNB1 in Paneth cells. c) Confocal images showing localization of the endogenous mTq2-CTNNB1 fusion protein in wholemount mTq2-Ctnnb1 mouse intestine. d) Confocal microscopy images showing live mTq2-CTNNB1 intestinal organoids for transmission and mTq2 channel. Top: overview of a complete organoid. Bottom: zoom of a crypt region. mTq2 signal is found at the adherens junctions but no nuclear accumulation is found in the Paneth cells (arrowheads).
To use the mTq2-CTNNB1 fusion protein as a direct reporter of WNT/CTNNB1 signalling in the mice, we turned to the intestine, as this tissue is well-studied in the Wnt signalling field (Perochon et al., 2018), with active WNT/CTNNB1 signalling controlling stem cell maintenance in the intestinal crypt. As previously reported in the literature (van Es et al., 2005; Van Noort et al., 2002), we detected CTNNB1 by immunohistochemistry at the adherens junctions of all epithelial cells and in the nucleus of the Paneth cells in the intestinal crypt in wildtype mice (Figure 7b). Wholemount confocal microscopy imaging of the endogenous mTq2 fluorescence signal in the intestine of heterozygous \( mTq2-Ctnnb1 \) mice, also clearly revealed CTNNB1 to be present in the adherence junctions. However, we did not detect nuclear accumulation of the fusion protein in Paneth cells (Figure 7c).

Our fusion protein allows imaging of signal transduction events in real-time. This remains challenging in whole tissues, but luckily the novel organoid technology has provided a suitable compromise to image live cells in a 3D context (Rios and Clevers, 2018). They are very accessible for live imaging at high resolution, while at the same time showing complex structures and different cell types. We established intestinal organoids from heterozygous \( mTq2-Ctnnb1 \) mice and grew these in glass-bottom plates to allow direct confocal imaging (Figure 7d). Again, the endogenous fluorescent signal at the adherens junctions was easily visualized, but again, no nuclear accumulation of CTNNB1 could be detected in the Paneth cells.

**Discussion**

Here, we describe the generation of \( mTq2-Ctnnb1 \) knock-in mice via CRISPR/Cas9 genome editing in zygotes. When we started this project, CRISPR/Cas was foreboded as a promising method for generating knock-in mice much faster and with a higher success rate than traditional targeting. It is generally perceived as a revolution in the genetic engineering field, but in practice remained challenging in mouse zygotes. Although it took a considerable investment of time and resources, we ultimately established an efficient protocol.

**Setting up the CRISPR toolbox**

Since our lab had no prior experience with CRISPR/Cas9 genome editing, we performed initial testing in HEK293A cells (Figure 1). We set up a pipeline to design gRNAs and test them for their capacity to create large deletions or indels in the \( CTNNB1 \) locus. The first specific genomic changes made were point mutations to create a constitutively active variant of CTNNB1 (S33Y and S45F). This was relatively efficient, reaching 14-17% successful targeting. If similar efficiencies would be
achieved for zygote targeting, it would be sufficient to inject zygotes for about two litters, in order to get a founder carrying a desired point mutation.

As proof-of-principle for knock-in experiments and to check if the endogenous fluorescent signal could be imaged to begin with, CTNNB1 was tagged with four different monomeric fluorescent proteins in HEK293A cells (Figure 2). The expression pattern of FP-CTNNB1 was as expected: most signal can be found at the adherens junctions and the signal is very low in the cytoplasm and nucleus in the absence of WNT pathway stimulation. Because of the low endogenous signal, finding cells under the microscope is challenging. This was further aggravated by the very low targeting efficiencies of a larger knock-in construct. Methods to increase HDR have been published, but available solutions often only increase efficiency by 2- to 10-fold (Devkota, 2018), which may not be sufficient to yield a big enough improvement in practice. To enrich for tagged cells, FACS sorting was performed to isolate them from the non-tagged population (Figure 3). This proved to be challenging as well, because the endogenous expression levels resulted in a fluorescent signal that was close to the detection limit.

**Challenges for quantitative cell biology studies**

Picking the correct cell line is important for any biological experiment, so also when the goal is to tag endogenous genes with CRISPR. Importantly, cells should be easy to transfect, have a working HDR machinery and express the protein of interest. In our case, the cells also needed to have a functional WNT/CTNNB1 signalling response. As we found out, for CRISPR/Cas9 mediated tagging the ploidy of the cell line is also an important factor to consider.

Most immortalized cell lines are not diploid but show a more complex – hypotriploid in the case of HEK293A – genome organisation. If more than two copies of a gene are present, the chances of successful homozygous tagging become smaller. Moreover, because CRISPR/Cas9 mediated cutting and repair is far more efficient than knocking in a specific sequence via HDR, any untagged alleles will frequently present with indels even if one or more other alleles are properly repaired. This complicates matters if it is the explicit goal of the study to leave endogenous gene expression and protein function undisturbed. For example, the use of fluorescent fusion proteins allows quantitative measurements of protein concentration, binding affinity to other (tagged) proteins and the size of protein complexes to be measured (Lippincott-Schwartz et al., 2001). But when it is unclear how many alleles are present and if only some of those are fluorescently tagged and others (potentially) disrupted, interpreting the data becomes challenging, especially since allele-specific expression can be variable per cell- or tissue type (Cowles et al., 2002). Ideally, one would always work with homozygously tagged cell
lines for each protein of interest. However, when studying several genes of interest in one cell line, this could become experimentally challenging because of the relative inefficiency of HDR. Therefore, it is important to consider using a cell line that is truly diploid or even (nearly) haploid, such as the Hap-1 cell line (Essletzbichler et al., 2014).

The mouse mammary gland epithelial cell line BC44 was chosen to optimize CRISPR/Cas9 mediated cutting and repair for the mouse CTNNB1 locus (Figure 4). Despite the low tagging efficiency in BC44 cells, FACS sorting and limiting dilution ultimately allowed us to establish clonal populations containing mTq-CTNNB1 and SGFP2-CTNNB1 tagged alleles. Surprisingly, all (12/12) SGFP2 clones were heterozygous, while for the mTq2 clones half (5/10) were homozygous. No differences were expected because all were tagged using the same Cas9/sgRNA plasmid DNA and the exact same sequence for the homology arms in the repair constructs. Moreover, the DNA sequence for SGFP2 and mTq2 differ only seven bases from each other. So why was such an apparent difference found? We hypothesize that this is probably due to a combination of our gating settings and the laser lines and filters of the FACS, which results in maximum sensitivity for SGFP2 (and other green fluorescent proteins). The blue channel, however, is suboptimal for activating and detecting the cyan fluorescent protein mTq2 (Figure 3d). Moreover, cells tend to show more autofluorescence in shorter (blue-shifted) wavelengths, so background levels can be higher as well (Aubin, 1979). Taking this together, we hypothesize that the sensitivity for the blue mTq2 signal is so low that heterozygously tagged cells were not gated as fluorescent, but remained hidden in the non-sorted population, resulting in the majority of the sorted cells being homozygous. In contrast, because of the inefficiency of scarless tagging and, as a result, the low chance of targeting all alleles in one cell, most green cells will be heterozygously tagged. In theory, this might be improved by further optimizing the gating strategy.

As alluded to above, heterozygously tagged clonal cell lines may be less suitable for quantitative cell biology using fluorescent microscopy: when the clone only has knock-in alleles, the fusion protein contributes 100% to signalling in the cells, but this is less clear when one or more untagged alleles remain present. Moreover, because NHEJ is more efficient than error-free HDR, there is a chance that the non-tagged alleles are not wildtype but have indels that affect expression or function of the non-tagged protein. It is therefore important to sequence all alleles separately. PCR mediated amplification of the Ctnnb1 locus followed by Sanger sequencing showed mixed reads for some of the heterozygous mTq2- and SGFP2-CTNNB1 BC44 cell lines. Indeed, when we performed metaphase spreads for BC44 cells, some of the spreads clearly showed this cell line to be hypotriploid as well (data not shown).
Creating \textit{mTq2-Ctnnb1} knock-in mice

It remains unclear how much can be learned from optimizing CRISPR/Cas9 tagging processes in cell lines for the purpose of creating knock-in mice via zygote injections. Ideally, a systematic analysis should be performed to compare different gRNAs and different delivery methods of CRISPR/Cas9 and repair templates. To test and compare cutting efficiencies a lot of zygotes are needed. Testing in cells might not be informative, because it is unclear if these results can be compared directly. For example, locus accessibility can differ.

Different gRNAs were used in our attempts to tag \textit{Ctnnb1} in mice (Table 1). Of note, we established knock-in BC44 cell lines using gRNAs 1 and 3 (Figure 4), but ultimately, we were only able to generate knock-in mice with a newly designed gRNA (gRNA 5). Importantly, all gRNAs used induce double-stranded DNA breaks within an 8bp distance to the start of the homology arms. Their proximity to the integration site should result in the highest HDR rates. HDR rates drop from 10−30\% depending on whether the gRNA is close (≤8 bases) to the integration site or further away (>8 bases) in iPSC and HEK293 cell lines (Liang et al., 2017b).

Our attempts with DNA repair plasmids with 800bp or 2000bp homology arms ultimately resulted in <2\% HDR efficiency (1/60), which is too low to be of practical use for a mouse knockout and transgenic core facility. Theoretically, chemical inhibitors of NHEJ could result in a relative increase in HDR rates, but this method is not without risk as the inhibitors could potentially disrupt repair processes essential for correct development of the zygote (Devkota, 2018). Therefore, they must be used with the utmost care and we preferred an approach to increase tagging efficiency without the need for chemical inhibitors. Ultimately, introducing Cas9/gRNA as a pre-assembled RNP together with an Lss oligo repair template with 120bp and 60bp homology arms allowed tagging of \textit{Ctnnb1} in such an efficient way that this would be the preferred method for targeting purposes in zygotes. Although we did not test this, the use of dsDNA repair templates might be equally efficient when combined with Cas9 protein as the Lss oligos used. However, cloning these big repair constructs is more cumbersome and time consuming. The Lss oligos can be ordered easily, or generated in house from dsDNA (Quadros et al., 2017), and therefore are the preferred repair template. Overall, the combination of a gRNA <8bp from the repair site, Cas9 RNP and LSS oligo works for effectively tagging the \textit{Ctnnb1} locus in zygotes.

Of note, integrations of seemingly random genomic regions were found in some blastocysts and mice, twice with the 800bp repair plasmid once with the Lss oligo repair template (Table 1; Figure 6). Both repetitive and non-repetitive sequences were found to be integrated. The flanking areas of the integrated sequences showed no homology to \textit{Ctnnb1}, and at most 1 or 2 basepairs of microhomology. Strikingly, both random and partial integrations of mTQ2 were only found when Cas9 mRNA was injected (4/151 = 2.6\%), but not when Cas9 protein was used (0/119). The time
of Cas9 activity in the zygotes might contribute to both random and successful, targeted integrations. However, these events should be further investigated to find the responsible mechanism.

**Lessons learned from tagging endogenous CTNNB1**

An important lesson that we learned from tagging CTNNB1 is that the endogenous expression of this protein is much lower than might be judged based on detection techniques that require amplification, such as immunohistochemistry. Next to its role in WNT signalling, CTNNB1 is part of the protein complex that forms the adherens junctions in the cell (Jamora and Fuchs, 2002). The mTq2-CTNNB1 fusion protein can indeed be detected in the plasma membrane of cells in the intestine (Figure 7). However, while immunohistochemistry shows accumulation of CTNNB1 in the nucleus of Paneth cells, this pattern is not observed for our fluorescent knock-in reporter (van de Wetering et al., 2002). Increased nuclear levels of CTNNB1 when WNT signalling is active are a dogma in the field, yet changes in CTNNB1 can be very subtle: while absolute levels can differ between cell types (Tan et al., 2012), as little as a 2-5 fold increase in the level of intracellular CTNNB1 can induce a robust TCF/LEF reporter response (Jacobsen et al., 2016). Since the endogenous CTNNB1 signal is relatively low, this increase might be too small to detect with our current setup. This discrepancy of the endogenous fluorescent signal should be further tested to check whether the lack of nuclear accumulation of the mTq2 signal is a result of the tagging or not. Amplification of the signal via antibody staining could provide answers as to whether the fusion protein does or does not accumulate in the nucleus at low levels. Moreover, the intestinal organoids can be used as relevant physiological context to study whether the fusion protein participates in a normal WNT response, because it is possible to both hyperactivate and inhibit WNT/CTNNB1 signalling by adding small chemical compounds to their culture medium and following the response of the fluorescent fusion protein (van de Moosdijk et al., 2020).

Although the fluorescent signal from the fusion protein is difficult to detect by eye, it can be imaged at a confocal microscope. For the FACS, the signal is close to the detection limit, making it challenging to distinguish fluorescent cells over wildtype. This depends not only on the gene expression levels, but also on the available laser lines, filters and sensitivity of the detectors in combination with the fluorescent protein chosen. Some other WNT pathway components, for example *Axin1* and *Axin2*, are expected to be expressed at much lower levels than CTNNB1, which could result in tagged alleles not being detectable by FACS or conventional fluorescence microscopy.

Because of the low endogenous signal, the choice of fluorescent protein is particularly important. When creating fusion proteins, monomeric FPs are required to avoid aggregation of the fusion proteins, which can seriously interfere with localization and biological function of the proteins under study. In our hands, of four
monomeric FPs tested in cell lines, mTq2 and SGFP2 were easier to image than SYFP2 and mCherry. However, novel monomeric FPs with all colours of the rainbow are being rapidly developed in several labs and might lead to a wider choice of suitable proteins in the future. Although brightness and stability are important factors, other variables should be considered as well: it is important to carefully select the best suited FP, depending on the types of experiments planned, the model system used, and technical properties or specifications of the available equipment. Consequently, selecting the optimal FP is a custom process for each project and experimental question.

Of course, the design of the fusion protein is important as well. We inserted a fluorescent protein at the N-terminus of CTNNB1, without the need for any linker sequences and with all 5’ and 3’ gene regulatory elements remaining intact. Moreover, there are studies that have created CTNNB1 fusion proteins with a 3xFlag (Choi et al., 2019), and GFP inserted at the N-terminus (Gagoski et al., 2016; Murase et al., 2002), respectively. Therefore, this N-terminal fusion protein should not show impaired activity. In both HEK293A and BC44 cell lines, the FP-CTNNB1 fusion protein correctly localizes at the adherens junctions and at very low levels in the cytoplasm, while not being detectable in the nucleus. This matches the expected pattern in the absence of WNT stimulation. Furthermore, in HEK293A cells we observed accumulation of mTq2-CTNNB1 in the cytoplasm and nucleus upon treatment with the GSK3-inhibitor CHIR99021. This confirms that the protein can still bind its interaction partners at the adherens junctions and that the destruction complex is able to destroy free-floating FP-CTNNB1. The viability of homozygous mTq2-Ctnnb1 mice further suggests that the fusion protein is fully functional: WNT signalling is essential for early development of the mouse embryo. In Ctnnb1 knock-out mice, a defect in anterior-posterior axis formation occurs and the embryos die around E6-7 (Huelsken et al., 2000). If WNT/CTNNB1 signalling would be impaired, homozygous mTq2-Ctnnb1 knock-in mice would not be expected to be viable.

In conclusion, mTq2-Ctnnb1 knock-in mice are healthy and express a bright fluorescent fusion protein to visualize endogenous CTNNB1 in tissues. The localization of CTNNB1 at the adherens junctions is as expected, but more experiments should be performed to determine whether the lack of nuclear accumulation in WNT/CTNNB1 responsive cells is a result of the tagging, or because of a difference in sensitivity of the endogenous fluorescent channel compared to the enzymatically enhanced immunohistochemistry signal.
Material & Methods

CRISPR/Cas9 constructs

gRNAs were designed using the MIT design tool from the Zhang lab (2014 – 2016, via http://crispr.mit.edu/) and CRISPOR (2017-2018, via http://crispor.tefor.net/). These tools were used to find gRNA sequences and identify putative off-target sites. BLAST analysis was performed to confirm uniqueness of gRNA sites in the genome. gRNAs were ordered as oligos from IDT. Complementary oligos were mixed in equimolar amounts, annealed at high temperature (~95-100°C) and allowed to cool slowly to room temperature. Subsequently, they were cloned into a Cas9 vector (pX330 and/or pX462, Addgene #42230 and #62987, respectively) linearized with BbsI. Ligations were performed using T4 DNA ligase (Thermo Scientific EL0014) in 10 μL reactions containing 1 μL enzyme, 20ng backbone, 1 μL oligo dilution (100μM dissolved in MQ) and 1 μL T4 ligase buffer. From the resulting plasmids, RNA could be transcribed using the T7 MEGashortscript kit (Fisher) according to the manufacturer’s instructions. Cas9 RNA was transcribed from the Cas9 vector using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Fisher) according to the manufacturer’s instructions. Cas9 protein was ordered from IDT (ALT-R).

Repair constructs

Repair plasmids were cloned via Gibson assembly (Gibson et al., 2009) in a pBluescript plasmid backbone linearized by SacI digestion. Homology arms were amplified from genomic DNA via overhang PCR. For hsCTNNB1, genomic DNA was isolated from HEK293a cells. For the experiments in BC44 cells genomic DNA was isolated from BC44 cells. For the experiments in zygotes, genomic DNA was isolated from FVB/N mice. The volume needed was calculated according to the Gibson protocol with a maximum volume of 10µl per assembly. Fragments were incubated 15 min at 50°C in molar ratios of 1:3 backbone:insert with 10µl Gibson Assembly Mastermix (2x): 8µl 5x ISO buffer, 10U/µl T5 exonuclease (NEB), 2U/µl Phusion DNA polymerase (Thermo Scientific) and 40U/µl Taq DNA ligase (NEB). After Gibson Assembly, each repair construct was thoroughly checked via sequencing.

Short single-stranded repair oligos (<100nt, 30-50nt homology) were ordered from IDT. For long single stranded (Lss) repair oligos (120/60nt asymmetric homology), a Long ssDNA Preparation Kit (Biodynamics) was used according to the manufacturer’s instructions.
Cell culture
HEK293A cells were cultured at 37°C and 5% CO₂ in DMEM medium supplemented with Glutamax and 10% FBS. Cells were transfected with PEI (Polyethylenimine, Polysciences, Omnilabo) dissolved in milliQ with a DNA:PEI ratio of 1:6. Medium was refreshed 4 to 6 hours after transfection.

BC44 cells (a gift from Marie-Ange Deugnier (Deugnier et al., 1999)) were cultured at 37°C and 5% CO₂ in RPMI 1650 with 2mM L-glutamine, 10% FBS and 5µg/ml Insulin (Sigma, human recombinant). Cells were transfected with Xfect transfection reagent (Clontech). Concentrations used: 0.3µl polymer per 1µg plasmid. Medium was refreshed 4 to 6 hours after transfection.

Isolation and culture of mouse intestinal organoids
Mouse intestinal organoid cultures were established as previously described (Sato et al., 2009). Crypts were isolated from the entire length of the small intestine of mTq2-CTNNB1 (HET) animals and used to establish individual organoid lines. Organoids were cultured at 37°C and 5% CO₂ in 10 µl Matrigel droplets (Corning) in culture medium containing advanced DMEM/F12 (ThermoFisher Scientific) supplemented with 100 U/ml Penicillin/Streptomycin (ThermoFisher Scientific), 2 mM Glutamax (ThermoFisher Scientific), 10mM HEPES (ThermoFisher scientific), 1x B27 supplement (ThermoFisher Scientific) and 1.25 mM N-acetylCysteine (Sigma Aldrich), freshly added EGF (50 ng/ml, PeproTech), recombinant murine Noggin (100 ng/ml, PeproTech) and recombinant murine R-spondin 1 (500 ng/ml, Sinobiological Inc.). For passaging, cell culture medium was removed and Matrigel was broken into small pieces by scraping, followed by vigorous pipetting with ice-cold advanced DMEM/F12. Crypts were centrifuged at 200g for 5 min at 4°C. The supernatant was carefully removed, the pellet was resuspended in Matrigel and plated on pre-warmed plates. Medium was refreshed every other day and organoids were split once a week in a 1:3 ratio.

Immunohistochemistry
4% PFA-fixed (Merck) and paraffin-embedded Balb/c small intestinal tissue was cut into 15 µm sections and floated on water. Tissues sections were picked up onto a superfrost plus slide (Thermo Scientific), deparaffinized, and then rehydrated. Following citrate-based antigen retrieval (pH 6.0), tissue sections were stained with an anti-beta-catenin antibody (mouse monoclonal, BD Bioscience, 61053, 1:800). Signal was detected using the Vecta Stain Elite ABC kit (used according to the manufacturer’s instructions) and DAB substrate. Sections were counterstained with 50% hematoxylin, dehydrated in a graded series of ethanol dilutions followed by Histoclear II (National Diagnostics), and mounted with a coverslip using Omnimount (National Diagnostics). Pictures were taken using an Axio scope A1 microscope with a Nikon Ri2 camera and NIS F freeware.
Confocal microscopy
For both cell lines, growth medium without Fenol Red was used to reduce background in microscopy experiments. For live cell imaging, cells were cultured on 25mm glass coverslips in 6-well plates. Right before imaging, medium was replaced with pre-warmed Microscopy Medium (137 mM NaCl, 5.4mM KCl, 1.8 mM CaCl2, 0.8mM MgCl2, 20mM glucose, and 20mM HEPES at PH = 7.4). CHIR99021 (BioVision) was dissolved in DMSO at 6 mM and added at described concentrations.

Images were made on a Nikon A1 confocal microscope, using a 63x oil immersion objective. mTq2 was imaged using a 457 nm laser, 457/514/561 nm dichroic mirror and bandpass (BP) 482/35 nm emission filter. SGFP2 was imaged with a 488 nm laser, 405/488 dichroic and BP535/70 nm emission filter. SYFP2 was imaged with 488 nm laser, combined with 457/514/561 nm dichroic and BP540/30 nm emission filter. For imaging mCherry, a 561 nm laser combined with a 405/488/561/640 nm dichroic was used and a BP595/50 nm emission filter.

For imaging tissues, samples were fixed 1 hour in 4% PFA (fresh) and cleared in glycerol. For this, the tissue is washed in series of 20/50/80% glycerol before being transferred to 100% glycerol. Once cleared, the tissue is mounted and imaged. Imaging was performed on a Leica SP8 confocal microscope with LasX software, using a 20x air and 40x oil objective. Excitation laser for mTq2 was 440nm. PMTs with settings 452-595 were used for detection.

For imaging organoids, samples were grown in 8-well glass-bottom µ slides (Ibidi) and imaged live at 37 °C. Imaging was performed on a Leica SP8 confocal microscope with LasX software, using a 20x air objective. Excitation laser for mTq2 was 440nm. PMTs with settings 452-600 were used for detection.

FACS analysis and single cell sorting
To prepare cells for FACS sorting, cells were washed with pre-cooled HBSS containing 2% FBS twice before resuspension in ice-cold HBSS with 2% FBS. Cells were kept on ice until sorting and filtered using a cell strainer. Cells were analysed and sorted with a BD FACS AriaIII. The sorting was based on manual gating to select for single cells and cells positive for fluorescence. Cells were sorted into their respective medium containing 5% Pen/Strep, either in a tube when a population was required to plate in a 12- or 6-well plate, or single cells directly into a 96-well plate, coated with 0.1% bovine gelatin (Sigma-Aldrich).

Isolation and analysis of genomic DNA
Isolation of genomic DNA was performed following the protocol from Laird et al (Laird et al., 1991). Regions of interest were amplified using PCR and subsequently sequenced via Sanger sequencing. Primers used for *hsCTNNB1*: 555 and 558 (supplementary table 2). For *mmCtnnb1*, primer 978 and 980 were used when
≤800bp homology arms were present in the repair construct. For the 2kb homology arm constructs, the primers used were 1440-1441. If these didn’t provide a product, the construct was checked by doing two PCR analyses with primers spaced closer, together spanning the entire repaired locus: 1440-673 and 978-1441 (supplementary table 2).

Sequence analysis was performed in ApE and the online tool Benchling.com. To scan for gRNA efficiency and detect indels, either a SURVEYOR assay (Qiu et al., 2004) was performed or the samples were run on a non-denaturing PAGE gel (Chen et al., 2012; Sambrook and Russell, 2001). Where indicated, the DNA sequences obtained as described above was analysed using the TIDE tool (Brinkman et al., 2014), available via tide.nki.nl.

RNA isolation and cDNA synthesis
Total RNA was isolated from confluent cell cultures using TRIZol reagent (Fisher Scientific) according to the manufacturer’s guidelines. Residual genomic DNA was digested by RQ1 RNase-free DNase treatment (Promega) according to the manufacturer’s instructions. The RNA concentration was determined using a Nanodrop spectrophotometer. Purity of all samples was assessed by the absorbance ratios of OD260/280 and OD260/230. cDNA was synthesized from 2µg RNA using SuperScript IV Reverse Transcriptase (Invitrogen) and Random Hexamers (Fisher Scientific), according to the manufacturer’s instructions. RiboLock RNase-inhibitor (Thermo Scientific) was added during the reverse transcriptase reaction. The resulting cDNA was diluted 10-fold for subsequent qRT-PCR analysis.

Quantitative Real-Time PCR analysis
qRT-PCR was performed using an Applied Biosystems 7500 Real-Time PCR System. PCR reactions (total 20 µl) were set up containing 13 µl RNAse-free H2O, 4 µl 5× HOT FIREPol® EvaGreen® qRT-PCR Mix Plus ROX (Solis Biodyne), 0.5 µl of each specific forward and reverse primer (10 μM stock) and 2 µl of diluted cDNA template. The reactions were set up in technical triplicates in 96-well PCR plates. One negative control (no-RT) reaction was included for each sample/primer combination. Thermal cycling was performed, starting with an initial step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. Each run was completed with a melting curve analysis. Primer sequences are listed in supplementary table 2.

Western Blot analysis
Confluent cells were harvested by lysis in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl, pH 8.0) supplemented with protease inhibitors (Roche). Protein concentration was determined using a Pierce BCA protein kit (BioRad), following the manufacturer’s instructions. Denatured
samples were run on a 10% SDS-PAGE gel and transferred to a 0.2µM nitrocellulose membrane (Biorad) for at 30V for 16 hours; or at 260mA for 3 hours, both at 4°C. The blot was blocked for 1 hour at room temperature in Odyssey Blocking Buffer (LI-COR) diluted 1:1 in TBS, followed by overnight incubation with primary antibody at 4°C. Primary antibodies were used to recognize GFP (1:1000, Thermo Fisher, A-6455), active CTNNB1 (1:1000, Cell Signalling, 8814), total beta-catenin (1:2000, BD Bioscience, G10153), and tubulin (1:1000, Sigma Aldrich Co, T9026). After incubation, blots were washed extensively with TBS-Tween (0.1%) before incubating with the secondary antibodies for 1 hour at room temperature. Secondary antibodies were IRDye 680LT anti-mouse (1:20.000, LI-COR) and IRDye 800CW anti-rabbit (1:20.000, LI-COR). Imaging was done with an Odyssey Fc Dual-Model Imaging System (LI-COR). Image analysis was performed using LI-COR Image Studio Lite software.

**Generation of a CRISPR knock-in mouse line**
Mice were generated at the NKI transgenic core facility. CRISPR components were injected into zygotes according to the concentrations listed in Table 1 and zygotes were checked for timely cell division O/N. Blastocysts were transferred into pseudopregnant foster mothers. Mosaic founders were identified by PCR using primers 978 and 980 (see supplementary table 2) and bred to wildtype FVB/N mice and the offspring was checked for germline transmission via genotyping PCR using Phire polymerase (initially performed using primers 672, 673 and 1783, later replaced by 3372/3374/3376, see supplementary table 2).

The line was transferred and maintained within the SILS Animal Facility. Breeding of mouse lines was performed in-house. Mice were housed in IVC cages on a 12h light/dark cycle and received food and water ad libitum. All experiments were performed in accordance with institutional and national guidelines and regulations. All experimental protocols were approved by the Animal Welfare Committee of the Netherlands Cancer Institute or the University of Amsterdam in the respective facilities.
An *mTq2-Ctnnb1* knock-in mouse via CRISPR/Cas9 genome editing

**Supplementary information**

**Supplementary table 1**: gRNA sequences

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**Supplementary table 2**: Primer sequences

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Chapter 6

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**Gibson primers:**

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<td>582 mTq2 FW + overhang</td>
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<td>583 mTq2 RV + overhang</td>
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**Supplementary table 3: repair sequences for point mutations**

Point mutation in magenta, bases altered to prevent gRNA re-cutting are depicted in blue, PAM sequence is underlined.

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<thead>
<tr>
<th>Repair Site</th>
<th>Repair Sequence</th>
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<td>S45F repair for gRNA 59 (RvA#576):</td>
<td>CCATTCTGGTGCCACTACCACAGCTCTTTTCTGAGTGGTAAGGGAAACCCTGAGGAAGAGGATGTGGATACCTCCCAAGTCCTGTATGAGTG GGAACAG</td>
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<tr>
<td>S45F repair for gRNA 61 (RvA#577):</td>
<td>CTACCTGGACTCTGGAATCCATTCTGGTGCCACTACCACAGCTCTTTTCTTAGCGGAAGGCAATCCTGAGGAAGAGGATGTGGATACCTCCCAAGTACCTGTACCTCCAAGT</td>
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<tr>
<td>S33Y repair for gRNA 63 (RvA#578):</td>
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<td>S33Y repair for gRNA 65 (RvA#579):</td>
<td>GCGGCTGTTAGTCACTGGCAGCAACAGTCTTTTAGATTATGGAATTCACAGTGGTGCACCTACCACAGCTCTTTTCTGAGTGTAAGGGCAATCCTG</td>
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</tbody>
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**Supplementary sequences**: full plasmid sequences for endogenous loci and repair templates are available via osf.io/43yj2.
Author contributions

AvdM, SdM, LK, IH and RvA conceived and designed the experiments. AvdM, SdM and LH performed the HEK293A and BC44 experiments. LK and IH oversaw the zygote injections. LK and AvdM analysed the blastocysts and knock-in mice. SdM and AvdM performed the mouse experiments. YvdG cultured and performed the intestinal organoid experiments. AvdM, SdM and LH performed imaging of the samples. AvdM and RvA analysed the data and wrote the manuscript.

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